Title:
Complementary roles for differential gene expression and differential exon use in the heat shock response of an intertidal copepod.

Authors:

Abstract:
Understanding the mechanisms by which organisms adapt to variation in temperature is key to explaining their distribution across environments and to predicting their response to changing climate. The cellular response to heat stress, heat shock response (HSR), is a highly conserved mechanism for coping with elevated temperatures which functions through the upregulation of molecular chaperones like heat shock proteins (HSPs). Recent studies have also shown cellular response to heat stress can be quantitative (changing the magnitude of expression) or qualitative (expressing different exons originating from the same gene). However, few studies have explored the time course of these two mechanisms in response to heat shock. We conducted a time-course experiment to examine the gene expression and exon usage changes in response to heat stress at four post-stress timepoints (30 minutes, 1 hour, 2 hours, 24 hours) in a splashpool copepod, *Tigriopus californicus*. We detected signatures of both gene expression and exon usage changes across all timepoints. The magnitude of this response was higher at timepoints closer to heat shock and decreased with time post-heat shock. Genes coding for heat shock proteins, cellular growth, and differentiation responded to heat stress predominantly through changing their expression levels, whereas genes coding for peptidases and chitin synthesis responded through changing expression levels and exon usage. Genes involved in cellular metabolism and cytoskeletal elements responded to heat shock primarily through changing exon usage. These ontology-specific response mechanisms provide new insights into temporal landscape of heat shock response in *Tigriopus* and highlight the need to integrate qualitative and quantitative changes in gene expression to fully understand the organismal response to heat stress.

Introduction:

An organism's ability to acclimate and adapt to temperature changes is crucial in determining its survival and, ultimately, the spatial distributions of species and populations. For most organisms, temperatures slightly above their optimum thermal range present a challenge to survival (Richter et al. 2010) because everything from rates of reactions in the cell to the behavior of the organism is affected by temperature (Pereira et al. 2017). Inside the cell, an

increase in temperature causes protein misfolding and the aggregation of misfolded proteins, eventually disrupting protein homeostasis, damaging the cytoskeleton, affecting the number and integrity of cellular organelles like mitochondria, and negatively affecting rates of transcription and translation as well as cell growth and development (Richter et al. 2010). Therefore, it is essential that organism's sense and rapidly respond to this cellular challenge to survive changes in temperature.

The cellular response to temperature stress, or the heat shock response (HSR), is widely conserved across the tree of life, but there is also variation within and across and species in this trait (Ritossa 1962; Jamrich et al. 1977; Kelley and Schlesinger 1978; Lemaux et al. 1978; Ashburner and Bonner 1979; McAlister and Finkelstein 1980; Dutton and Hofmann 2009; Henkel et al. 2009; Schoville et al. 2012; Barreto et al. 2018; Li et al. 2018; Alagar Boopathy et al. 2022; Earhart et al. 2022). One of the primary response mechanisms to heat stress is increased expression of molecular chaperones such as Heat Shock Proteins (HSPs), which help to prevent and repair the damage caused by heat stress. Comparisons of HSR across species have also revealed that the timing and duration of the response varies across species and even within populations within the same species (Snutch and Baillie 1983; Peter and Candido 2002; Han and Lee 2006; Velichko et al. 2013; Jayaraj et al. 2020). However, it is also possible that cells could be responding to heat stress via changes in gene isoforms along with changes in the magnitude of gene expression for a gene – this could be achieved by choosing different combinations of exons in stress versus non-stressed conditions (i.e., alternative splicing or differential exon usage)(Healy and Schulte 2019). This combination of qualitative and quantitative responses to stress has been reported in several organisms (De Nadal et al. 2011; Kornblihtt et al. 2013; Laloum et al. 2018; Xia et al. 2018; Chaudhary et al. 2019; Healy and Schulte 2019; Tan et al. 2019; Zhang et al. 2019; Salisbury et al. 2021; Thorstensen et al. 2021). Heat stress may shift expression towards more thermally stable isoforms of key enzymes, contributing to inter and intra-specific variation in heat tolerance (Scafaro et al. 2016; Nagarajan et al. 2025). More broadly, mounting evidence for the role of alternative splicing in heat stress responses underscores the need to include analyses that consider changes in gene expression as well as changes in exon usage (Ling et al. 2021). Similarly, understanding the temporal landscape of changes in gene expression and exon usage, and their potential interactions at several time points post-heat stress, can also be helpful to understand the time course of heat stress responses.

Here, we investigate the relative importance of differential exon usage and differential gene expression during heat shock responses, using the common splash pool copepod, *Tigriopus californicus* as our study system. With a habitat that spans >27° of latitude from Baja, California to Alaska, *T. californicus* tolerates a wide range of latitudinal and thermal variation (Burton and Lee 1994). Within a site, the splash pools that *T. californicus* inhabit also experience a wide range of temperature variation over short periods of time – with temperatures fluctuating >20˚C during the course of several hours on a sunny day in addition to significant seasonal variability throughout much of its range. *T. californicus* also has a short generation time, is easy to maintain in the lab, and shows population specific variation in heat tolerance (Kelly et al. 2012; Barreto et al. 2018). Moreover, the mechanistic basis of heat tolerance as a trait has been widely studied in Tigriopus (Schoville et al. 2012; Kelly et al. 2013; Graham and Barreto 2019;

Harada et al. 2019), but very few studies have focused on the changes in exon use in response to heat stress or the time course of gene expression and exon usage changes after experiencing heat stress. To address this gap in knowledge, we performed a time course experiment consisting of four time points post heat stress (30 mins, 1 hour, 2 hours, 24 hours) to identify the how and when exons usage and gene express change over time following heat stress. We predicted that: i) changes in exon usage, in addition to gene expression, contribute to the heat shock response in *T. californicus*, and ii) gene expression and exon usage exhibit a relation to time in response to heat stress.

Methods:

Animal collection and maintenance:

We collected *Tigriopus californicus* copepods from Strawberry Hill, Oregon (44°15′ N, 124°06′ W) in 2024. We established cultures in the lab from these wild-caught individuals and maintained animals following protocols described in Kelly et al. (2012). Briefly, copepods were maintained at 20°C and at 34 ppt salinity under a 12 hour light / 12 hour dark cycle and fed a diet of ground spirulina fish food *ad libitum* for 2-3 generations before beginning the experiment.

Experimental design:

Our experimental design consisted of four time points post heat shock (30 minutes, 1 hour, 2 hours, and 24 hours), with three replicate experimental groups for each time period.For each treatment, 50-60 adult copepods were haphazardly chosen from the established lab cultures and transferred into a 50 mL falcon tube containing sea water at 34 ppt salinity. For heat shock treatments, tubes were exposed to a sub-lethal temperature of 34°C for 1 hour (Kelly et al, 2012) in a water bath and then returned to the ambient temperature of 20°C until they were flash frozen in liquid nitrogen at their respective time points post heat shock (Fig. 1A). The control tube was kept at 20°C for the duration of heat shock before being flash frozen in liquid nitrogen. The flash frozen samples were stored in -80°C for RNA extractions.

RNA extraction and sequencing:

We extracted total RNA from flash frozen copepods using a combination of TRIzol (Invitrogen; catalog no. 15596026) and the Qiagen RNAeasy Plus kit (Qiagen, catalog no. 74134). Briefly, copepods were homogenized using a TissueRuptor II (Qiagen, catalog no. 9002755) before we followed steps 1-7 of the TRIzol protocol, followed by steps 4-11 of RNAeasy Plus kit protocol. Total RNA extracted from a total of 15 ((4 timepoints + 1 control) x 3) samples were sent to Novogene Corporation Inc. at Sacramento, California, where RNA quality control was confirmed using a 2100 Agilent Bioanalyzer on a Eukaryote Total RNA Nano chip and non-directional libraries were produced using poly-A tail selection. The resulting 15 libraries were sequenced on NovaSeq X Plus, with 150-bp paired-end reads. We removed adapter sequences using Trimmomatic (Bolger et al. 2014), and we used the program FASTQC (Andrews, 2010) to ensure that all of the readsconsidered for downstream analysis had quality scores of at least 35. The reads were then mapped to the *T. californicus* reference genome (Barreto et al. 2018) using STAR rna-seq aligner (version 2.6.0a) (Dobin et al., 2013). Reads were mapped to gene

features with the options (--quantMode GeneCounts --outFilterScoreMinOverLread 0.50 --outFilterMatchNminOverLread 0.50) to adjust for poly-A tail contamination (Sirovy et al. 2021), which generates a count matrix using ReadsPerGene.out.tab output. Transcripts per million (TPM) values used for visualizing our RNASeq dataset were generated using the RSEM package (Li and Dewey 2011). All downstream analyses were performed in R version 4.0.3 software (R Core Team 2021).

Differential gene expression analysis:

We removed lowly expressed genes, which were defined as genes that did not have at least 10 counts in 25% of our samples (Brown et al. 2025) from our dataset. This retained 15052 genes that were input to DESeq2 (v 1.24.0) (Love et al. 2014) to perform pairwise comparisons of each time point relative to controls, obtain a list of differentially expressed genes (DEG) for each time point, and calculate false discovery rates (FDR) using the Benjamini–Hochberg method (Benjamini and Hochberg 1995). Genes with an adjusted p-value cutoff of < 0.05 and a log2foldchange of > 1 (for upregulated genes) or < -1 (for downregulated genes) were considered to be differentially expressed. Gene ontology enrichment was performed using GO_MWU (Wright et al. 2015), using the Fisher's Exact Test ($p < 0.05$) and the scripts available at: https:// github.com/z0on/GO_MWU/blob/master/GO_MWU.R.

Differential exon usage analysis:

We used DEXSeq v1.22.0 (Anders et al. 2012) to calculate differential exon usage (DEU), following the protocols described in the package manual (available at http://bioconductor.org/packages/release/bioc/html/DEXSeq.html). As changes in exon usage arising from alternative splicing and usage of alternative transcription sites are counted as exon changes by DEXSeq,, we refer to all DEXSeq results as differential exon usage to account for both of these possibilities, as stated in the DEXSeq manual. Briefly, we first created a flattened GFF (general feature format file) for *T.californicus* using ENSEMBL annotations (Tcal_SD_v2.1, accession number: GCA_007210705.1). The file collapses all transcripts produced from a gene into a single, 'flattened' gene model with non-overlapping exons (counting bins). Reads were mapped to these counting bins, and differential exon usage was calculated as difference in the ratios of number of reads mapping to an individual exon and the number of genes mapping to all other exons of the same gene. We calculated differential exon usage for each timepoint using pairwise comparisons with control samples and calculated false discovery rates using Benjamini-Hochberg method (Benjamini and Hochberg 1995). Genes with an adjusted p-value of < 0.05 were considered to be genes showing differential exon usage.

Before performing GO analysis, the gene names in DEXSeq output files were first converted to NCBI gene name format, because our GO analysis pipeline (GO_MWU (Wright et al. 2015)) uses annotations in NCBI format. We merged the NCBI and ENSEMBL GFF files for *T.californicus* based on genomic coordinates to produce a consensus file containing gene names for each gene in both formatting styles. This also allowed us to compare DEXSeq results to DEG results. Gene ontology enrichment for genes showing differential exon usage was performed using GO_MWU (Wright et al. 2015), using the Fisher's Exact Test ($p < 0.05$). The R

package ggvenn was used to identify the overlap between genes showing differential gene expression and differential exon usage.

Results:

Overall, across timepoints, the response to heat shock consisted of both differential gene expression (DEG) and differential exon usage (DEU), and the number of DEGs and DEU genes decreased with time post heat shock (Table 1). We observed the highest number of DEGs (1530) and DEU genes (1296) at 30 minutes post heat shock and the lowest number of DEGs (432) and DEU genes (305) at 24 hours post heat shock. Among DEGs, more genes increased their expression in response to heat shock: 84%, 82%, 64%, and 53 % at 30 mins, 1 hour, 2 hour and 24 hours post heat-shock, respectively. Our Venn diagram comparisons revealed that a modest number of genes responded to heat-shock by changing their expression and their exon usage (hereafter referred as DEG + DEU, Fig. 1B), but this number was dwarfed by the larger subset of genes that showed only one response mechanism (DEG or DEU). Broadly, genes related to heat shock proteins and cell growth responded to heat shock predominantly by changing their expression (Fig. 2, 3), while genes related to metabolism and cytoskeletal proteins responded primarily by changing their exon usage (Fig. 4 and 5). Genes coding for peptidases, hydrolases, and chitin synthesis and breakdown responded to heat-shock by changing their expression as well as exon usage (Fig. 6 and 7).

Discussion:

Our data highlight the importance of differential exon usage (DEU) and differential gene expression (DEG) as two important, and largely complementary, cellular mechanisms involved in response to thermal stress. Differential exon usage offers the opportunity to diversify the available transcriptome and proteome independent of changes in the expression levels of a gene. By including DEU analyses in our study, we were able to identify several hundred additional genes that responded to thermal stress solely through changing their exon usage and these would not be captured by DEG analysis, alone. While most transcriptomic studies focus on differential gene expression in response to stress, our results underscore the importance of considering differential exon usage in addition to gene expression in order to gain a comprehensive understanding of the changes occurring in the cellular landscape of *Tigriopus* in response to thermal stress. We also identified several genes that responded to thermal stress by changing their exon usage and expression levels across our experimental timepoints, highlighting the interactive roles of these two mechanisms in thermal stress response. The smaller magnitude of genes showing both DEU and DEG was also observed in other study systems in response to environmental stress (Jakšić and Schlötterer 2016; Jacobs and Elmer 2021; Ren et al. 2022; Liu et al. 2024). To our knowledge, this is the first study investigating the changes in exon usage and gene expression patterns in *T. californicus* across a series of time points covering both immediate and prolonged response to thermal stress.

Genes coding for peptidases and hydrolases (i.e., enzymatic degradation of proteins) responded to thermal stress through changes in exon usage and gene expression levels (Fig. 6). Expression levels of carboxypeptidases, which can break down proteins by cleaving the peptide bond at carboxy terminus, increased at 30 minutes and 1 hour post-heat shock, followed by a decrease at 2 hours and 24 hours post heat shock. On the other hand, aminopeptidases, which break down proteins at the amino-terminus, and metallopeptidases, which require a metal ion for their peptidase activity, were upregulated from 30 minutes to 2 hours post-heat shock. Expression levels of genes coding for dipeptidases, which break down pairs of amino acids (Rawlings and Bateman 2019)., showed a decrease in response to heat shock across all timepoints Taken together, these expression patterns suggest that peptidases are an important component of heat shock response in *T. californicus*. Their presence across several time points indicates that, at cellular level, thermal stress negatively affects protein folding in *T.californicus*, leading to aggregation of mis-folded proteins in cells, which are then presumably degraded by these peptidases. As the metabolic cost of breaking down misfolded proteins and remaking them is higher than repairing them (Richter et al. 2010), the presence of peptidases in the heat shock response could mean that after experiencing thermal stress, *T. californicus* has large quantities of irreversibly damaged proteins that need to be degraded. Similarly, the decrease in expression levels of peptidases after 2 hours post heat-shock also suggests how long these irreversibly damaged proteins were present in the cell. Once these proteins are no longer present in the cell, it makes intuitive sense that the cells would cease to upregulate peptidases. Differential exon usage of peptidases also showed similar variation based on their mode of action and time post-heat shock. Genes coding for both carboxy and amino peptidases showed DEU across all timepoints, whereas dipeptidases showed DEU only at 1, 2, and 24 hours post heat shock.

We observed a similar pattern of changes in DEU and DEG responses in genes associated with chitin (Fig. 7). Chitin-related genes, such as chitin deacetylase, have been reported to be upregulated in response to temperature and hypoxia in *Tigriopus*, although their function remains unexplored (Andriot, 2024; Harada et al. 2019; Healy and Burton 2023; Schoville et al. 2012). Because chitin is an essential component of the copepod exoskeleton, microscopic examination of stressed copepods may prove helpful to identify cuticle remodeling that could be occurring in response to temperature.

On the other hand, genes coding for heat shock proteins (HSP) and cell development responded to thermal stress predominantly through changing their expression levels (Fig. 1). Several members of the HSP family including hsp70, hsp16, and dnaJ, showed increased expression levels at all timepoints post heat-shock. We also observed paralog specific differences in expression levels in HSPs. Out of six genes coding for hsp70, three showed increased expression at all timepoints, whereas the other three were upregulated at a specific time point post-heat shock. Only a small subset of HSPs (hsp83, hspb1, and hsp16) showed DEG and DEU responses. Interestingly, paralogs of dnaJ that showed DEU activity were not upregulated in response to heat shock. The role of HSPs in heat-shock response as molecular chaperons that prevent protein aggregation is a widely conserved response to heat shock across the kingdom of life (Morimoto 1998; Feder and Hofmann 1999; Sørensen et al. 2003).

Previous studies in the *Tigriopus* system have also identified an upregulation in HSPs an hour after heat-shock (Schoville et al. 2012; Harada and Burton 2020; Tangwancharoen et al. 2020). Our time point data provides additional temporal context to this upregulation – we now know that HSPs are upregulated as early as 30 minutes afterheat shock and continue to be upregulated as late as 24 hours post-heat shock. Results from our DEU analysis for HSPs reveal isoform-specific responses which were not detected in our DEG analysis and are an excellent example where DEU and DEG complement each other in the global response to thermal stress in *Tigriopus*.

Along with upregulation of HSPs, the reduction of cell growth and proliferation is another hallmark of the heat shock response (Richter et al. 2010). Thermal stress negatively affects several key processes occurring in the nucleus and arrests the cell cycle, leading to a pause in cell growth and proliferation. We see similar trends in our DEG results, which show downregulation of transcription factors like HOX, SOX, and FOX, which are integral to cell growth and differentiation processes (Kamachi and Kondoh 2013). Interestingly, we observed this downregulation only until 2 hours post-heat shock. By 24 hours after the shock, many of these genes were not downregulated, indicating a possible recovery to non-stressed cellular state by that time. Similar to HSPs, we observe very few instances of DEU activity in these genes, and there was no overlap between genes showing DEG and DEU activity.

Finally, we also observed a subset of genes that responded to heat-shock predominantly through DEU. These included genes involved in cytoskeletal elements (e.g., actin, tubulin) and metabolism (ATP synthase, pyruvate kinase, Acetyl CoA). Few genes from both of these functional categories were differentially expressed, and there was no overlap between differential expression and differential exon usage in these genes. Thermal stress is known to cause disruption of actin and tubulin networks in the cell (Welch and Suhan 1985, 1986; Toivola et al. 2010). Thermal stress also adversely affects energy production in the cell, as it decreases the number of mitochondria (Lambowitz et al. 1983; Patriarca and Maresca 1990). We detected several genes related to ATP synthesis and metabolism in our exon usage analysis, and a few in our DEG analysis. While a surprising result, the presence of cytoskeleton and metabolic genes in the DEU category could be an indicator of a switching out mechanism wherein exons that lead to heat-stable isoforms are favored to maintain the integrity of the cytoskeleton and help sustain energy production during heat stress.

Our observations of both DEG and DEU responses in several genes present an intriguing puzzle regarding the mechanistic decisions made in *T. californicus* in response to thermal stress. Exon switching offers the advantage of generating multiple protein isoforms with distinct functions from a single gene, which could be particularly beneficial under stress conditions. In contrast, increasing gene expression demands more energy, as it involves both transcription and translation. Understanding how cells balance these strategies — taking into account their metabolic costs and the functional versatility enabled by exon switching — could provide valuable mechanistic insights into adaptation to thermal stress. Our study is also one of the first to report the time course of transcriptomic response to heat stress in *T. californicus*.

Understanding the chronology of these cellular biological responses is essential for understanding how an organism recovers from stress, and findings from a non-model study can contribute to expanding our broader knowledge of heat shock responses across diverse taxa. Incorporating DEU analyses alongside DEG analyses revealed several genes that respond to heat stress through either DEU alone or a combination of DEU and DEG. Identities of these novel target genes could be valuable for future studies aimed at understanding the molecular mechanisms of heat tolerance in *T. californicus* and other species.

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Conflict of interest:
The authors declare no conflict of interests.

Data availability:
The DOI for the data files associated with this publication is: to be completed after manuscript is accepted for publication.

References:

Alagar Boopathy LR, Jacob-Tomas S, Alecki C, Vera M. 2022. Mechanisms tailoring the expression of heat shock proteins to proteostasis challenges. Journal of Biological Chemistry 298:101796.

Anders S, Reyes A, Huber W. 2012. Detecting differential usage of exons from RNA-Seq data. Nat Prec.

Andrews S. n.d. FastQC: A Quality Control Tool for High Throughput Sequence Data [Online]. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/. .

Andriot M. 2024. Coping mechanisms : the stress-induced regulatory landscape of the intertidal copepod, Tigriopus californicus.

Ashburner M, Bonner JJ. 1979. The induction of gene activity in drosophila by heat shock. Cell 17:241–54.

Barreto FS, Watson ET, Lima TG, Willett CS, Edmands S, Li W, Burton RS. 2018. Genomic signatures of mitonuclear coevolution across populations of Tigriopus californicus. Nat Ecol Evol 2:1250–57.

Benjamini Y, Hochberg Y. 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B: Statistical Methodology 57:289–300.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–20.

Brown KT, Dellaert Z, Martynek MP, Durian J, Mass T, Putnam HM, Barott KL. 2025. Extreme Environmental Variability Induces Frontloading of Coral Biomineralisation Genes to Maintain Calcification Under pCO_2 Variability. Molecular Ecology 34:e17603.

Burton RS, Lee BN. 1994. Nuclear and mitochondrial gene genealogies and allozyme polymorphism across a major phylogeographic break in the copepod Tigriopus californicus. Proc Natl Acad Sci USA 91:5197–5201.

Chaudhary S, Khokhar W, Jabre I, Reddy ASN, Byrne LJ, Wilson CM, Syed NH. 2019. Alternative Splicing and Protein Diversity: Plants Versus Animals. Front Plant Sci 10:708.

De Nadal E, Ammerer G, Posas F. 2011. Controlling gene expression in response to stress. Nat Rev Genet 12:833–45.

Dutton JM, Hofmann GE. 2009. Biogeographic variation in Mytilus galloprovincialis heat shock gene expression across the eastern Pacific range. Journal of Experimental Marine Biology and Ecology 376:37–42.

Earhart ML, Blanchard TS, Strowbridge N, Bugg WS, Schulte PM. 2022. Gene expression and latitudinal variation in the stress response in Fundulus heteroclitus. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 268:111188.

Feder ME, Hofmann GE. 1999. HEAT-SHOCK PROTEINS, MOLECULAR CHAPERONES, AND THE STRESS RESPONSE: Evolutionary and Ecological Physiology. Annu Rev Physiol 61:243–82.

Graham AM, Barreto FS. 2019. Novel microRNAs are associated with population divergence in transcriptional response to thermal stress in an intertidal copepod. Molecular Ecology 28:584–99.

Han M-J, Lee SY. 2006. The *Escherichia coli* Proteome: Past, Present, and Future Prospects. Microbiol Mol Biol Rev 70:362–439.

Harada AE, Burton RS. 2020. Consequences of *HSF* knockdown on gene expression during the heat shock response in *Tigriopus californicus*. Journal of Experimental Biology jeb.208611.

Harada AE, Healy TM, Burton RS. 2019. Variation in Thermal Tolerance and Its Relationship to Mitochondrial Function Across Populations of Tigriopus californicus. Front Physiol 10:213.

Healy TM, Burton RS. 2023. Differential gene expression and mitonuclear incompatibilities in fast- and slow-developing interpopulation *Tigriopus californicus* hybrids. Molecular Ecology 32:3102–17.

Healy TM, Schulte PM. 2019. Patterns of alternative splicing in response to cold acclimation in fish. Journal of Experimental Biology jeb.193516.

Henkel S, Kawai H, Hofmann G. 2009. Interspecific and interhabitat variation in hsp70 gene expression in native and invasive kelp populations. Mar Ecol Prog Ser 386:1–13.

Jacobs A, Elmer KR. 2021. Alternative splicing and gene expression play contrasting roles in the parallel phenotypic evolution of a salmonid fish. Molecular Ecology 30:4955–69.

Jakšić AM, Schlötterer C. 2016. The Interplay of Temperature and Genotype on Patterns of Alternative Splicing in *Drosophila melanogaster*. Genetics 204:315–25.

Jamrich M, Greenleaf AL, Bautz EK. 1977. Localization of RNA polymerase in polytene chromosomes of Drosophila melanogaster. Proc Natl Acad Sci USA 74:2079–83.

Jayaraj GG, Hipp MS, Hartl FU. 2020. Functional Modules of the Proteostasis Network. Cold Spring Harb Perspect Biol 12:a033951.

Kamachi Y, Kondoh H. 2013. Sox proteins: regulators of cell fate specification and differentiation. Development 140:4129–44.

Kelley PM, Schlesinger MJ. 1978. The effect of amino acid analogues and heat shock on gene expression in chicken embryo fibroblasts. Cell 15:1277–86.

Kelly MW, Grosberg RK, Sanford E. 2013. Trade-Offs, Geography, and Limits to Thermal Adaptation in a Tide Pool Copepod. The American Naturalist 181:846–54.
Kelly MW, Sanford E, Grosberg RK. 2012. Limited potential for adaptation to climate change in a broadly distributed marine crustacean. Proc R Soc B 279:349–56.
Kornblihtt AR, Schor IE, Alló M, Dujardin G, Petrillo E, Muñoz MJ. 2013. Alternative splicing: a pivotal step between eukaryotic transcription and translation. Nat Rev Mol Cell Biol 14:153–65.
Laloum T, Martín G, Duque P. 2018. Alternative Splicing Control of Abiotic Stress Responses. Trends in Plant Science 23:140–50.
Lambowitz AM, Kobayashi GS, Painter A, Medoff G. 1983. Possible relationship of morphogenesis in pathogenic fungus, Histoplasma capsulatum, to heat shock response. Nature 303:806–8.
Lemaux PG, Herendeen SL, Bloch PL, Neidhardt FC. 1978. Transient rates of synthesis of individual polypeptides in E. coli following temperature shifts. Cell 13:427–34.
Li A, Li L, Wang W, Song K, Zhang G. 2018. Transcriptomics and Fitness Data Reveal Adaptive Plasticity of Thermal Tolerance in Oysters Inhabiting Different Tidal Zones. Front Physiol 9:825.
Li B, Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 12:323.
Li BJ, Zhu ZX, Qin H, Meng ZN, Lin HR, Xia JH. 2020. Genome-Wide Characterization of Alternative Splicing Events and Their Responses to Cold Stress in Tilapia. Front Genet 11:244.
Ling Y, Mahfouz MM, Zhou S. 2021. Pre-mRNA alternative splicing as a modulator for heat stress response in plants. Trends in Plant Science 26:1153–70.
Liu Y, Xia X, Ren W, Hong X, Tang X, Pang H, Yang Y. 2024. Alternative splicing perspective to prey preference of environmentally friendly biological agent Cryptolaemus montrouzieri. BMC Genomics 25:967.
Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550.
McAlister L, Finkelstein DB. 1980. Heat shock proteins and thermal resistance in yeast. Biochemical and Biophysical Research Communications 93:819–24.
Morano KA, Grant CM, Moye-Rowley WS. 2012. The Response to Heat Shock and Oxidative Stress in *Saccharomyces cerevisiae*. Genetics 190:1157–95.
Morimoto RI. 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev 12:3788–96.
Nagarajan R, Kahlon KS, Mohan A, Gill KS. 2025. Tandemly duplicated Rubisco activase genes of cereals show differential evolution and response to heat stress. Plant Mol Biol 115:10.
Patriarca EJ, Maresca B. 1990. Acquired thermotolerance following heat shock protein synthesis prevents impairment of mitochondrial ATPase activity at elevated temperatures in Saccharomyces cerevisiae. Experimental Cell Research 190:57–64.
Pereira RJ, Sasaki MC, Burton RS. 2017. Adaptation to a latitudinal thermal gradient within a widespread copepod species: the contributions of genetic divergence and phenotypic plasticity. Proc R Soc B 284:20170236.
Peter E, Candido M. 2002. The Small Heat Shock Proteins of the Nematode Caenorhabditis elegans: Structure, Regulation and Biology. In: Arrigo A-P, Müller WEG, editors. Small Stress Proteins. Progress in Molecular and Subcellular Biology Berlin, Heidelberg: Springer Berlin Heidelberg. p. 61–78.
Rawlings ND, Bateman A. 2019. Origins of peptidases. Biochimie 166:4–18.

Ren Y, Tian Y, Mao X, Wen H, Qi X, Li J, Li J, Li Y. 2022. Acute hypoxia changes the gene expression profiles and alternative splicing landscape in gills of spotted sea bass (Lateolabrax maculatus). Front Mar Sci 9:1024218.

Richter K, Haslbeck M, Buchner J. 2010. The Heat Shock Response: Life on the Verge of Death. Molecular Cell 40:253–66.

Ritossa F. 1962. A new puffing pattern induced by temperature shock and DNP in drosophila. Experientia 18:571–73.

Salisbury SJ, Delgado ML, Dalziel AC. 2021. Alternative splicing: An overlooked mechanism contributing to local adaptation? Molecular Ecology 30:4951–54.

Scafaro AP, Gallé A, Van Rie J, Carmo-Silva E, Salvucci ME, Atwell BJ. 2016. Heat tolerance in a wild *Oryza* species is attributed to maintenance of Rubisco activation by a thermally stable Rubisco activase ortholog. New Phytologist 211:899–911.

Schoville SD, Barreto FS, Moy GW, Wolff A, Burton RS. 2012. Investigating the molecular basis of local adaptation to thermal stress: population differences in gene expression across the transcriptome of the copepod Tigriopus californicus. BMC Evol Biol 12:170.

Sirovy KA, Johnson KM, Casas SM, La Peyre JF, Kelly MW. 2021. Lack of genotype-by-environment interaction suggests limited potential for evolutionary changes in plasticity in the eastern oyster, *Crassostrea virginica*. Molecular Ecology 30:5721–34.

Snutch TP, Baillie DL. 1983. Alterations in the pattern of gene expression following heat shock in the nematode *Caenorhabditis elegans*. Can J Biochem Cell Biol 61:480–87.

Sørensen JG, Kristensen TN, Loeschcke V. 2003. The evolutionary and ecological role of heat shock proteins. Ecology Letters 6:1025–37.

Tan S, Wang W, Tian C, Niu D, Zhou T, Jin Y, Yang Y, Gao D, Dunham R, Liu Z. 2019. Heat stress induced alternative splicing in catfish as determined by transcriptome analysis. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 29:166–72.

Tangwancharoen S, Burton R. 2014. Early life stages are not always the most sensitive: heat stress responses in the copepod Tigriopus californicus. Mar Ecol Prog Ser 517:75–83.

Tangwancharoen S, Semmens BX, Burton RS. 2020. Allele-Specific Expression and Evolution of Gene Regulation Underlying Acute Heat Stress Response and Local Adaptation in the Copepod *Tigriopus californicus*. Journal of Heredity 111:539–47.

Thorstensen MJ, Baerwald MR, Jeffries KM. 2021. RNA sequencing describes both population structure and plasticity-selection dynamics in a non-model fish. BMC Genomics 22:273.

Toivola DM, Strnad P, Habtezion A, Omary MB. 2010. Intermediate filaments take the heat as stress proteins. Trends in Cell Biology 20:79–91.

Velichko AK, Markova EN, Petrova NV, Razin SV, Kantidze OL. 2013. Mechanisms of heat shock response in mammals. Cell Mol Life Sci 70:4229–41.

Welch WJ, Suhan JP. 1985. Morphological study of the mammalian stress response: characterization of changes in cytoplasmic organelles, cytoskeleton, and nucleoli, and appearance of intranuclear actin filaments in rat fibroblasts after heat-shock treatment. The Journal of cell biology 101:1198–1211.

Welch WJ, Suhan JP. 1986. Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. The Journal of cell biology 103:2035–52.

Wright RM, Aglyamova GV, Meyer E, Matz MV. 2015. Gene expression associated with white syndromes in a reef building coral, Acropora hyacinthus. BMC Genomics 16:371.

Xia JH, Li HL, Li BJ, Gu XH, Lin HR. 2018. Acute hypoxia stress induced abundant differential expression genes and alternative splicing events in heart of tilapia. Gene 639:52–61.

Yamauchi Y, Cooper PR, Shimizu E, Kobayashi Y, Smith AJ, Duncan HF. 2020. Histone Acetylation as a Regenerative Target in the Dentine-Pulp Complex. Front Genet 11:1.

Zhang M, Trushina NK, Lang T, Hahn M, Pasmanik-Chor M, Sharon A. 2023. Serine peptidases and increased amounts of soluble proteins contribute to heat priming of the plant pathogenic fungus *Botrytis cinerea*. mBio e01077-23.

Zhang X, Yuan J, Zhang X, Liu C, Xiang J, Li F. 2019. Genome-Wide Analysis of Alternative Splicing Provides Insights Into Stress Response of the Pacific White Shrimp Litopenaeus vanname. Front Genet 10:845.

Table 1: Number of DEGs and DEUs detected for each experimental time point.

Comparisons	Upregulated DEGs	Downregulated DEGs	Total DEGs	Number of DEU genes
CTRL vs 30mins	1298	236	1543	1296
CTRL vs 1 hour	1156	243	1399	956
CTRL vs 2 hours	824	459	1283	891
CTRL vs 24 hours	230	203	433	305

Figure 1: A) Schematic of the experimental design. B) Venn diagrams showing overlap between DEGs and DEUs at each experimental timepoint. C) Bar plot showing log2foldchange of a gene (hsp83) that changed gene expression and exon usage in response to heat shock. The grey bars correspond to gene expression change (log2fold values) calculated from DESeq2 and the blue and green bars represent two exons of this gene that showed differential usage (log2fold values calculated from DEXSeq). D) Bar plot showing log2foldchange of a gene (tubulin alpha chain-like) that only changed exon usage in response to heat shock. The blue and green bars represent two exons of this gene that showed differential usage (log2fold values calculated from DEXSeq). E) Bar plot showing log2foldchange of a gene that only changed gene expression in response to heat shock. The grey bars indicate gene expression change (log2fold values) calculated from DESeq2.

Figure 2: Heatmaps of genes coding for heat shock proteins that show significantly differential gene expression (DEGs) and differential exon usage (DEUs) in response to heat shock across four experimental timepoints (X-axis). The left side of the heatmap shows DEGs color coded based on their log2fold change values obtained from DESeq2 and the right side shows DEU genes color coded based on their log2fold change values from DEXSeq analysis.

Figure 3: Heatmaps of genes coding for cell growth and development that show significantly differential gene expression (DEGs) and differential exon usage (DEUs) in response to heat shock across four experimental timepoints (X-axis). The left side of the heatmap shows DEGs color coded based on their log2fold change values obtained from DESeq2 and the right side shows DEU genes color coded based on their log2fold change values from DEXSeq analysis.

Figure 4: Heatmaps of genes coding for cytoskeletal elements that show significantly differential gene expression (DEGs) and differential exon usage (DEUs) in response to heat shock across four experimental timepoints (X-axis). The left side of the heatmap shows DEGs color coded based on their log2fold change values obtained from DESeq2 and the right side shows DEU genes color coded based on their log2fold change values from DEXSeq analysis.

Figure 5: Heatmaps of genes involved in metabolic processes that show significantly differential gene expression (DEGs) and differential exon usage (DEUs) in response to heat shock across four experimental timepoints (X-axis). The left side of the heatmap shows DEGs color coded based on their log2fold change values obtained from DESeq2 and the right side shows DEU genes color coded based on their log2fold change values from DEXSeq analysis.

Figure 6: Heatmaps of genes coding for peptidases that show significantly differential gene expression (DEGs) and differential exon usage (DEUs) in response to heat shock across four experimental timepoints (X-axis). The left side of the heatmap shows DEGs color coded based on their log2fold change values obtained from DESeq2 and the right side shows DEU genes color coded based on their log2fold change values from DEXSeq analysis.

Figure 7: Heatmaps of genes involved in chitin synthesis and breakdown that show significantly differential gene expression (DEGs) and differential exon usage (DEUs) in response to heat shock across four experimental timepoints (X-axis). The left side of the heatmap shows DEGs color coded based on their log2fold change values obtained from DESeq2 and the right side shows DEU genes color coded based on their log2fold change values from DEXSeq analysis.

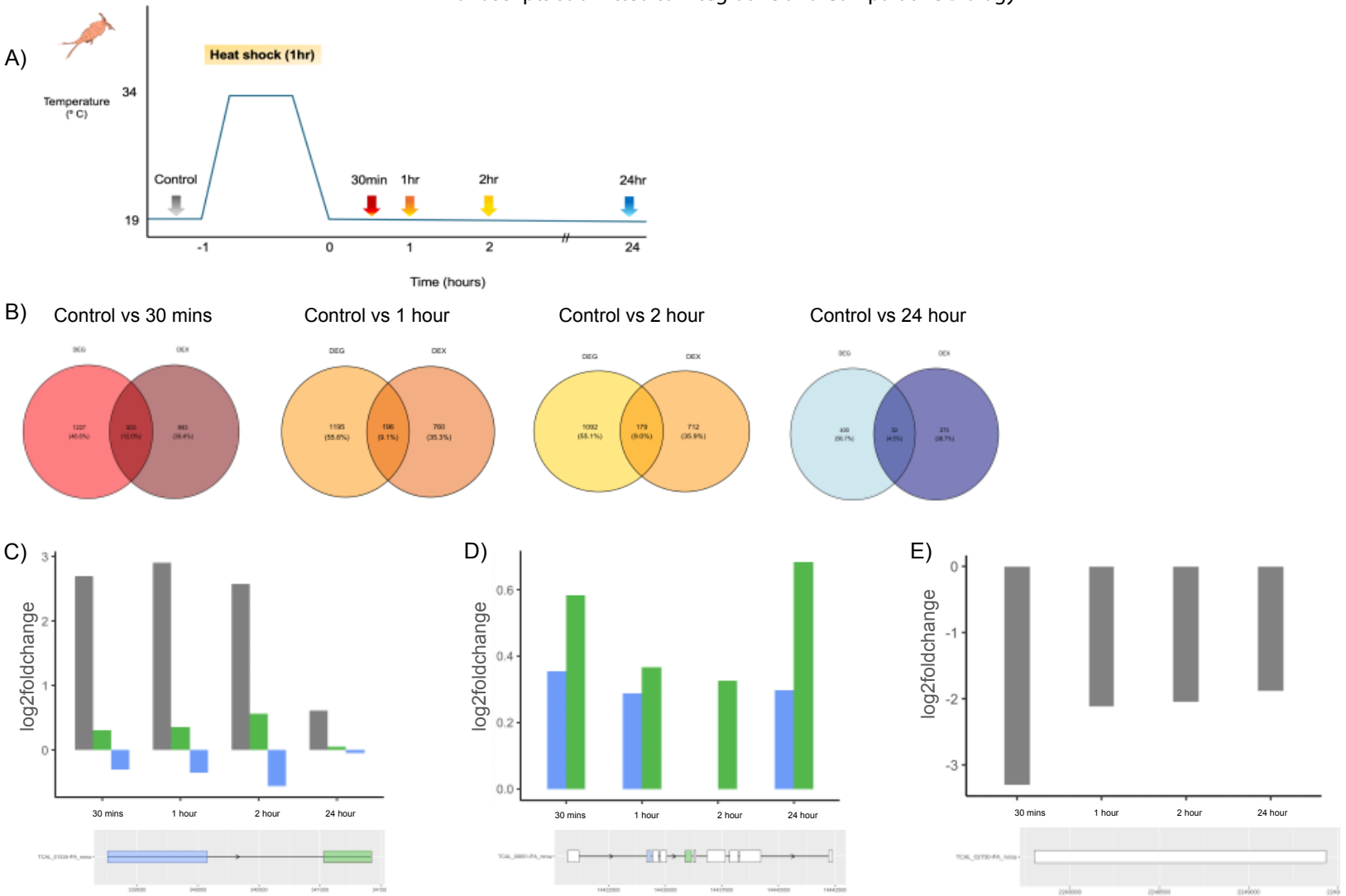
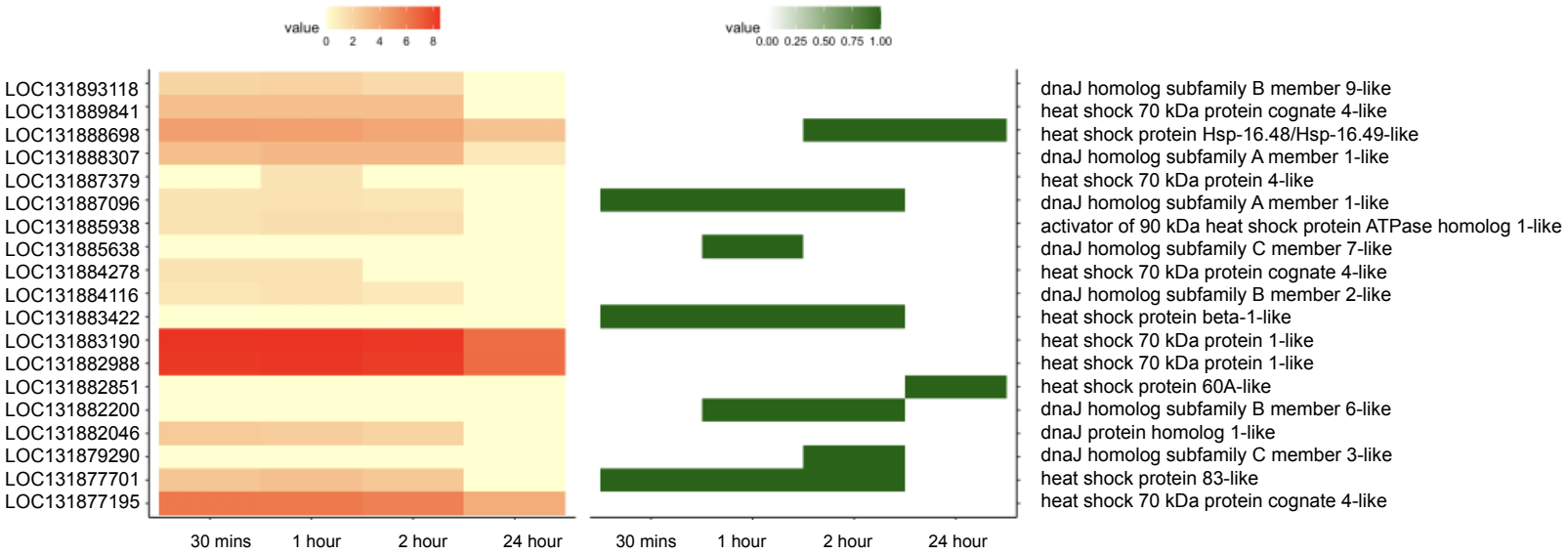


Figure 1: A) Schematic of the experimental design. B) Venn diagrams showing overlap between DEGs and DEUs at each experimental timepoint. C) Bar plot showing log2foldchange of a gene (hsp83) that changed gene expression and exon usage in response to heat shock. The grey bars correspond to gene expression change (log2fold values) calculated from DESeq2 and the blue and green bars represent two exons of this gene that showed differential usage (log2fold values calculated from DEXSeq). D) Bar plot showing log2foldchange of a gene (tubulin alpha chain-like) that only changed exon usage in response to heat shock. The blue and green bars represent two exons of this gene that showed differential usage (log2fold values calculated from DEXSeq). E) Bar plot showing log2foldchange of a gene that only changed gene expression in response to heat shock. The grey bars indicate gene expression change (log2fold values) calculated from DESeq2.



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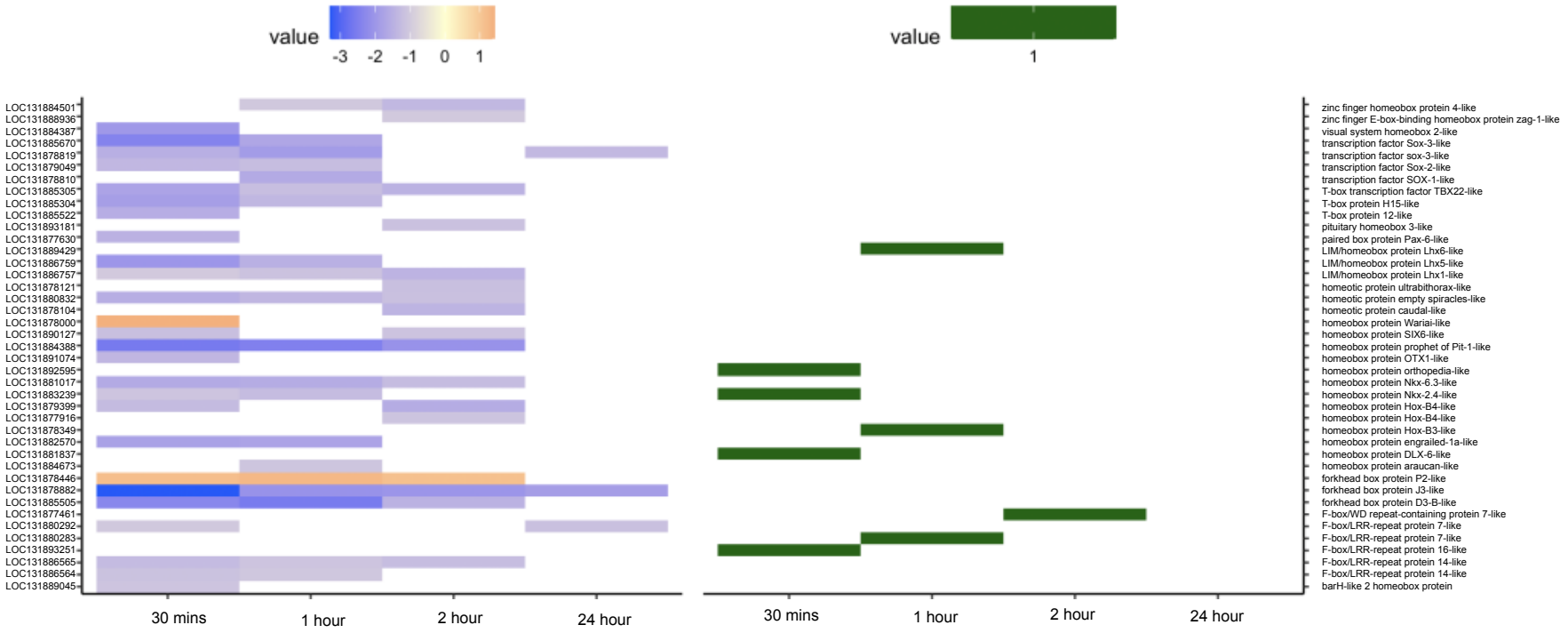

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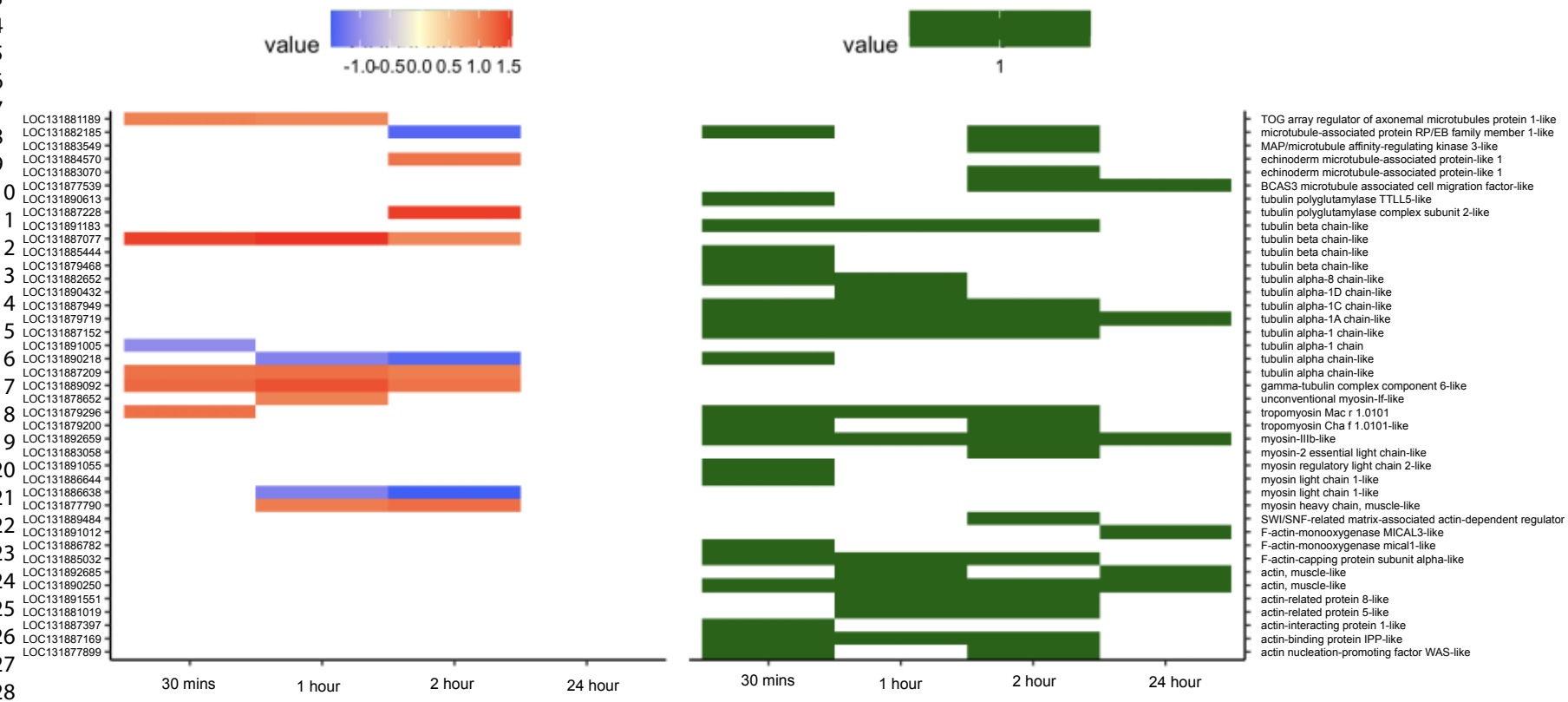

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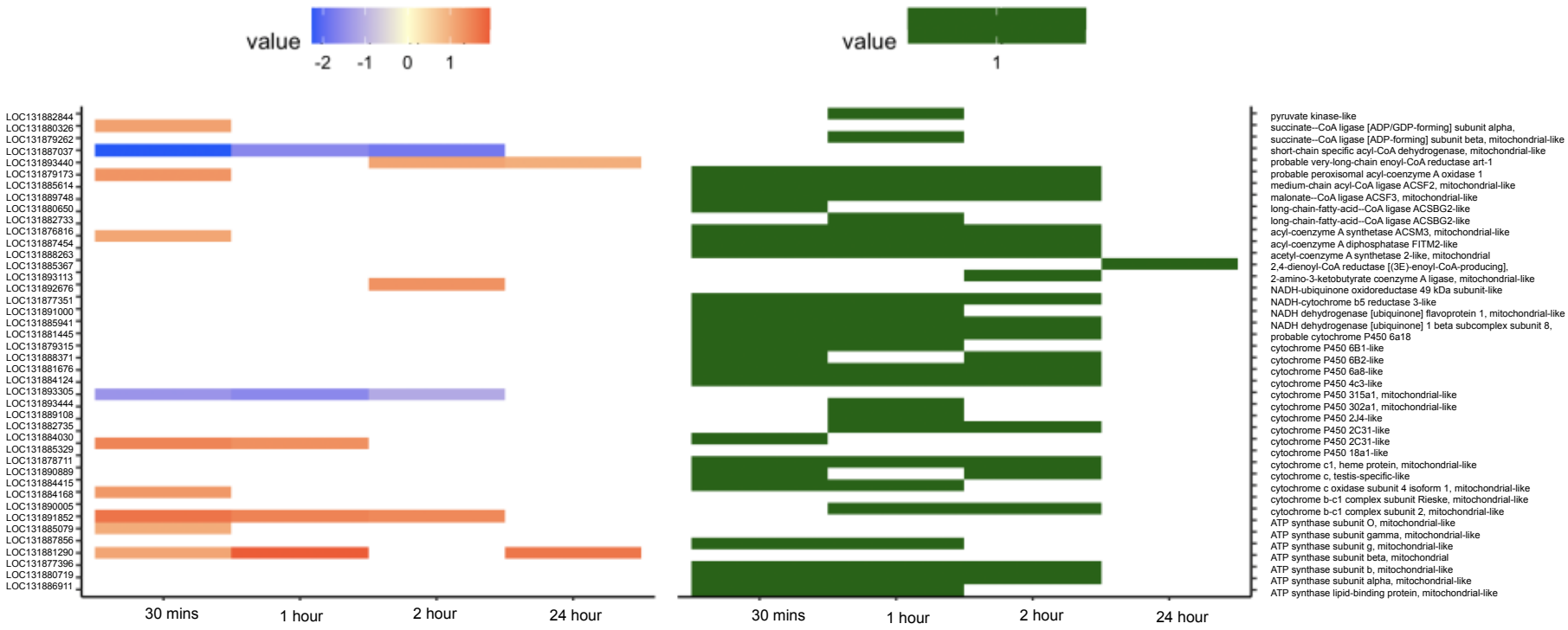


Figure 5: Heatmaps of genes involved in metabolic processes that show significantly differential gene expression (DEGs) and differential exon usage (DEUs) in response to heat shock across four experimental timepoints (X-axis). The left side of the heatmap shows DEGs color coded based on their log2fold change values obtained from DESeq2 and the right side shows

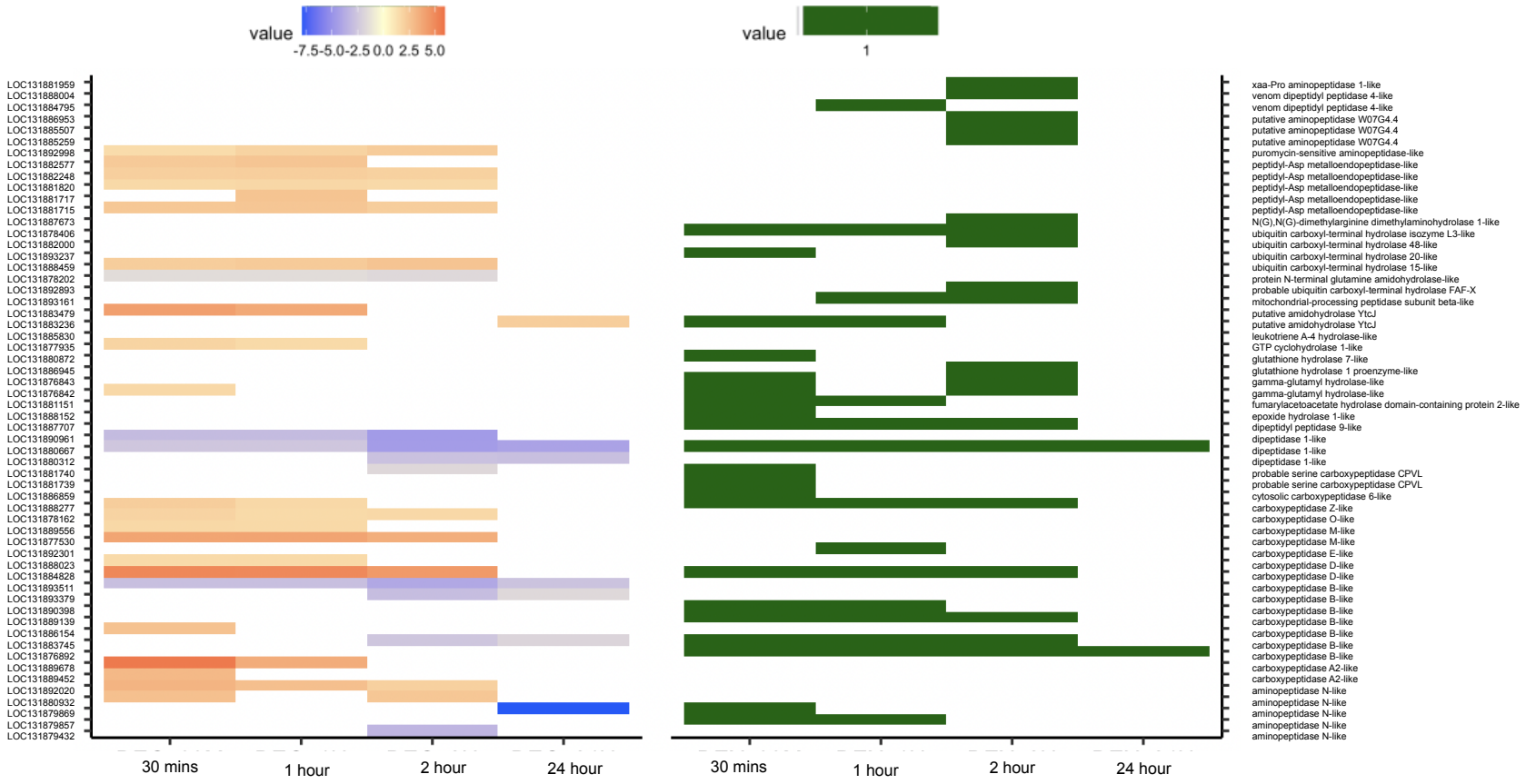
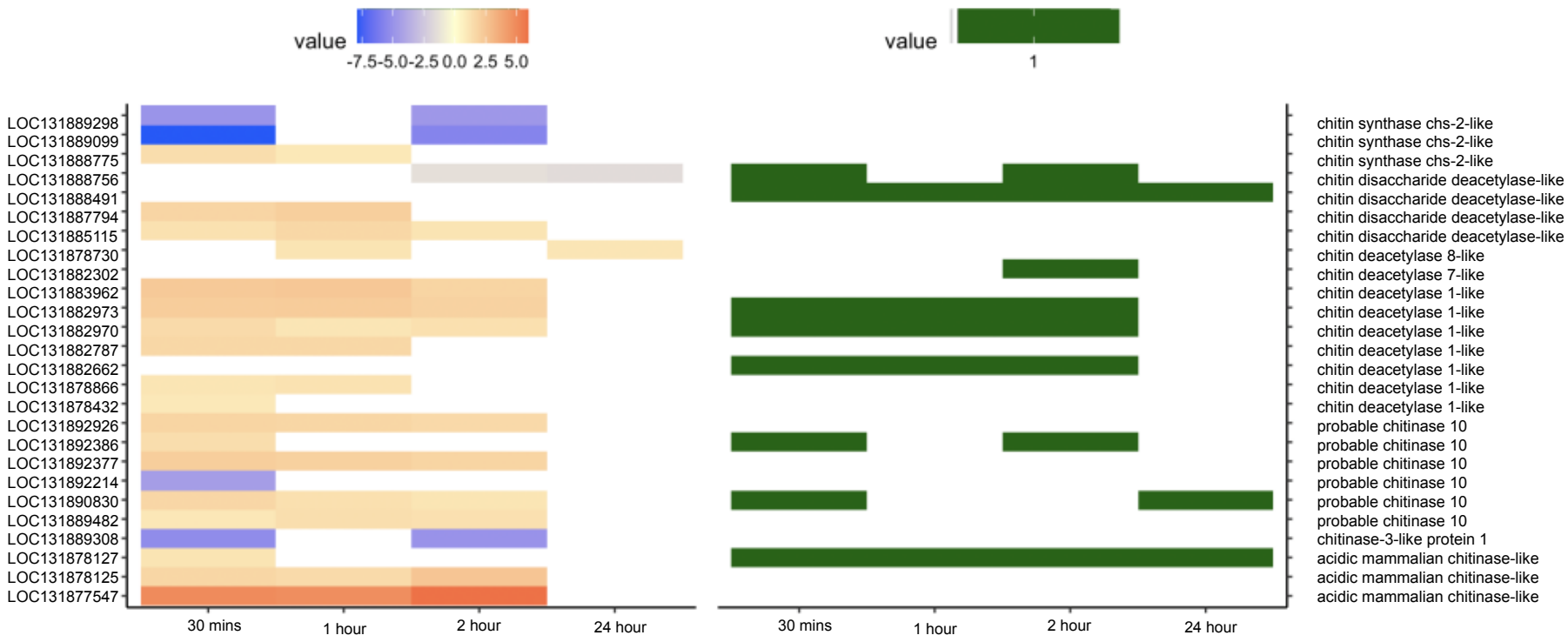


Figure 6: Heatmaps of genes coding for peptidases that show significantly differential gene expression (DEGs) and differential exon usage (DEUs) in response to heat shock across four experimental timepoints (X-axis). The left side of the heatmap shows DEGs color coded based on their log2fold change values



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